REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
12/2008	Journal Article	Oct 2005-Oct 2007

4. TITLE AND SUBTITLE
Viral Association with the Elusive Rickettsia of Viper Plague from Ghana, West Africa

5a. CONTRACT NUMBER
N/A

5b. GRANT NUMBER
N/A

5c. PROGRAM ELEMENT NUMBER
62202F

6. AUTHOR(S)
Johnathan L. Kiel,* Yvette Gonzalez,* Jill E. Parker,* Carrie Andrews,* Dominique Martinez,[⌂] Nathalie Vachiery,[⌂] and Thierry Lefrancois[⌂]

5d. PROJECT NUMBER
7757

5e. TASK NUMBER
P4

5f. WORK UNIT NUMBER
01

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Air Force Materiel Command Counterproliferation Branch
Air Force Research Laboratory Brooks City-Base, Texas 78235
711 Human Performance Wing
Human Effectiveness Directorate
Biosciences and Protection Division

8. PERFORMING ORGANIZATION REPORT NUMBER
AFRL-HE-BR-JA-2007-0017

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Materiel Command
Air Force Research Laboratory
711 Human Performance Wing
Human Effectiveness Directorate
Biosciences and Protection Division
Counterproliferation Branch
Brooks City-Base, Texas 78235

10. SPONSOR/MONITOR'S ACRONYM(S)
711 HPW/RHPC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-HE-BR-JA-2007-0017

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution A. Approved for Public release; distribution unlimited. Public Affairs case file no. 07-220, 1 Jun 07.

13. SUPPLEMENTARY NOTES:
Article published in the animal Biodiversity and Emerging Diseases Journal, N.Y. Academy of Sciences, Vol 1149: 318-321 (2008)

14. ABSTRACT
We previously reported a rickettsial heartwater-like disease in vipers from Ghana that resembled heartwater in its gross lesions, that was apparently transmitted by ticks (*Aponomma* and *Amblyomma*), and responded clinically favorably to early treatment with tetracycline. Cell culture showed consistent cytopathic effects in bovine endothelial cells, viper cells, and mouse cells, and inhibition of cytopathic effect by tetracycline *in vitro*. A type D retrovirus was observed in vacuoles in all infected cells. The virus and rickettsia infection was associated with transfer of cytopathic effect, regardless of cell species. Close association of virus and rickettsia may indicate a dual infection etiology of viper plague.

15. SUBJECT TERMS
Viper plague, rickettsia, heartwater, Ehrlichia, type D retrovirus

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr Johnathan Kiel
U	U	U	SAR	4	19b. TELEPHONE NUMBER *(include area code)* NA

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. 239.18

THIS PAGE INTENTIONALLY LEFT BLANK

Viral Association with the Elusive Rickettsia of Viper Plague from Ghana, West Africa

Johnathan L. Kiel,[a] Yvette Gonzalez,[a] Jill E. Parker,[a] Carrie Andrews,[a] Dominique Martinez,[b] Nathalie Vachiéry,[b] and Thierry Lefrançois[b]

[a]*Human Effectiveness Directorate, Air Force Research Laboratory, Brooks City-Base, Texas, USA*

[b]*CIRAD-EMVT, Domaine de Duclos, Prise d'Eau, Guadeloupe, French West Indies*

We previously reported a rickettsial heartwater-like disease in vipers from Ghana that resembled heartwater in its gross lesions, was apparently transmitted by ticks (*Aponomma* and *Amblyomma*), and responded clinically favorably to early treatment with tetracycline. Cell culture showed consistent cytopathic effects in bovine endothelial cells, viper cells, and mouse cells, and inhibition of cytopathic effect by tetracycline *in vitro*. A type D retrovirus was observed in vacuoles in all infected cells. The virus and rickettsia infection was associated with transfer of cytopathic effect, regardless of cell species. Close association of virus and rickettsia may indicate a dual infection etiology of viper plague.

Key words: **viper plague; heartwater; ehrlichia; type D retrovirus**

Introduction

Viper plague is a tick-borne (*Aponomma latum*) rickettsial disease that resembles heartwater (caused by *Ehrlichia ruminantium*), which was introduced into the United States in 2002 by Gaboon vipers (*Bitis gabonica gabonica* and *Bitis gabonica rhinoceros*) imported from Ghana, West Africa.[1] The infection spread in the United States through a private collection, and subsequently infected and killed at least 22 snakes, including Gaboon vipers, rhinoceros vipers (*Bitis nasicornis*), a Sri Lankan cobra (*Naja naja polycellata*), a monocellate cobra (*Naja naja kaouthia*), a black-necked cobra (*Naja nigricollis*), and bullsnakes (*Pituophis melanoleucus sayi)*. The outbreak was stopped by treatment with acaricide (permethrin) and oral treatment with tetracycline prior to the onset of clinical signs (those treated after onset were not responsive and died). Polymerase chain reaction (PCR), using pCS20 primers, yielded products from original postmortem tissues and subsequent viper cell cultures. Sequences from viper plague had high homology to each other but differed significantly from those produced by authentic *E. ruminantium*.

Materials and Methods

Cell Cultures and Electron Microscopy

Cell lines were obtained from the American Type Culture Collection, Rockville, MD: Russell's viper spleen epithelial cells (VSW; ATCC CCL-120), Russell's viper heart fibroblast cells (ATCC CCL-140), and bovine pulmonary artery endothelium endothelial cells (ATCC CRL-1733).

Molecular Biology

DNA was extracted with a QIAamp DNA mini kit (Ref:51306; Qiagen, Valencia,

Address for correspondence: Johnathan L. Kiel, AFRL/HEPC, 2486 Gillingham Drive, Brooks City-Base, TX 78235-5107. Voice: +1-210-536-3583; fax: +1-210-536-4716. Johnathan.Kiel@brooks.af.mil

Animal Biodiversity and Emerging Diseases: Ann. N.Y. Acad. Sci. 1149: 318–321 (2008).
doi: 10.1196/annals.1428.092

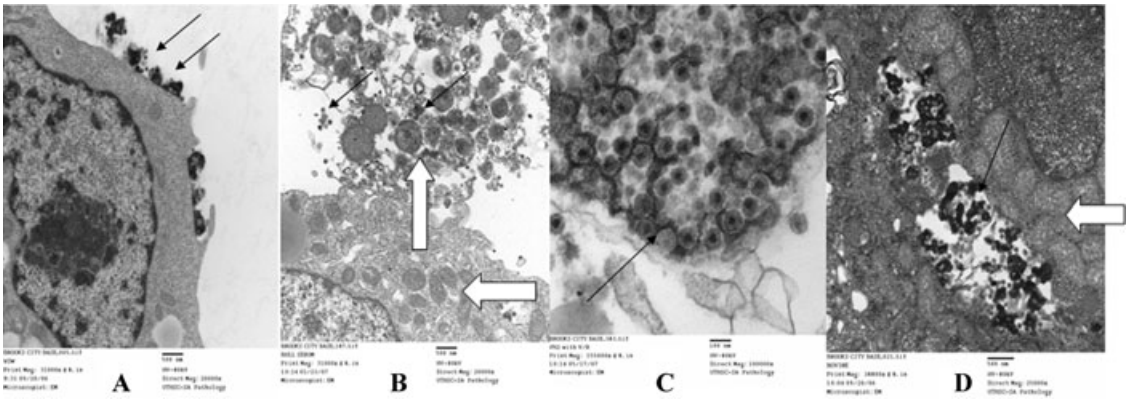

Figure 1. Virus (slim black arrows) in Russell's viper spleen cells (VSW; **A** and **B**) and heart cells (VH2; **C**), infected with serum from bull snake, and in bovine endothelial cells (BECs; **D**). Vertical open white arrow shows virus associated with rickettsia. Horizontal open white arrows indicates mitochondria.

CA). AB128 and AB129 were used as internal primers of nested PCR.[2] Nested primers for the detection of gene pCS20 were forward primer AB128: 5′ACTAGTAGAAATTGCACAATC TAT 3′; the external reverse primer AB130: 5′ACTAGCAGCTTTCTGTTCAGCTAG 3′; and the internal reverse primer AB129: TGATAACTTGGTGCGGGAAATCCTT-3′ AB. The primer AB130 was selected in a region without SNP polymorphism after alignment of the corresponding genome fragment of three different viper plague strains. After preheating the DNA at 94°C for 3 min, the first round of PCR with AB128 and AB130 primers was conducted under the following conditions: 35 cycles of a 45-s denaturation at 94°C, a 45-s annealing at 50°C, a 45-s elongation at 72°C, and a final 10-min extension at 72°C. One microliter of pure or 1/10 dilution of the PCR product from the first round was submitted to a second round of PCR with AB128 and AB129 primers consisting of 35 cycles of a 45-s denaturation at 94°C, a 45-s annealing at 55°C and 45 s at 72°C, followed by a 10-min extension step at 72°C. DNA purified from cell cultures of *E. ruminantium* (Gardel) was used as the positive control, and the negative control was water. Generalized primers for GAG and POL were used to search for unknown retroviruses (cDNA) in the infected cell DNA preparations as previously reported.[3]

Results

Culture and Microscopy of Rickettsia and Retrovirus

Figure 1 shows the retrovirus and rickettsia co-infecting viper cells (VSW and VH2) and bovine endothelial cells (BECs), attached to membrane structures, and released from cytopathic vacuoles in aggregations. In all the cell types inoculated, there were various degrees of apoptosis, necrosis, and formation of fusion plaques. The addition of the antibiotic tetracycline inhibited cytopathic effect versus the control in all cell cultures (data not shown). Also, after culture of serum of a secondarily infected bull snake, a greater concentration of the rickettsial organism was generated in subsequent cultures.

Ehrlichia ruminantium and Retroviral PCR

Nested PCR pCS20 AB128/129/130 primers gave strong positive results with DNA extracted from infected cells in the Guadeloupe laboratory (Fig. 2), but much weaker results with DNA extracted from cells in the AFRL (U.S.) laboratory and then shipped to Guadeloupe for testing (data not shown).

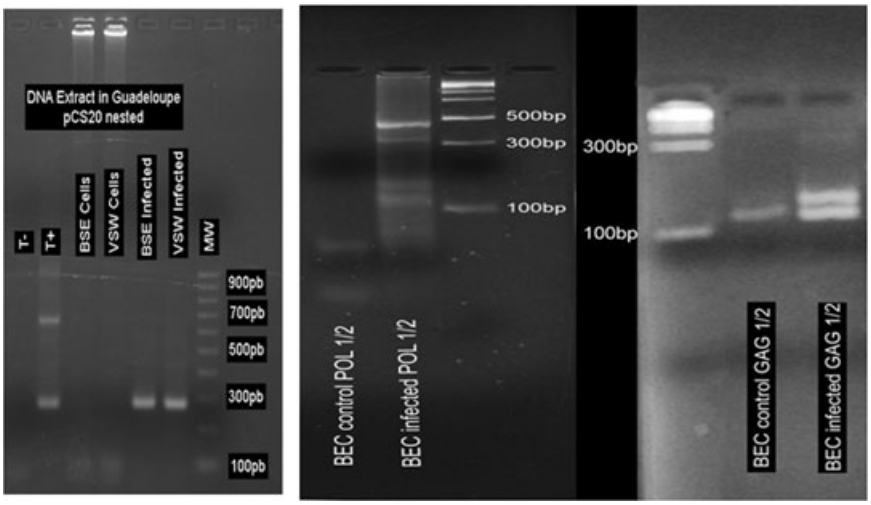

Figure 2. Polymerase chain reaction (PCR) products from control and infected cells. The left panel shows nested primer, pCS20 AB128/129/130, results, positive signals of infected cells. BSE (French abbrev.) = bovine endothelial cell (BEC); VSW = viper cells; T− = negative control; T+ = positive control, authentic *Ehrlichia ruminantium* DNA. The right panels show POL and GAG primer PCR products from DNA of control and infected BECs.

PCR products of GAG and POL primers, from DNA of control and infected BECs, respectively, are shown in Figure 2. Although a band in the ~150 bp region is noted in the control cells with the GAG primers (probably from endogenous retroviral sequences in the host DNA), alternate bands in the infected cells for both primers are present that are only associated with viper plague microbial infection.

Discussion

Any proposal to ban importation of snakes or other reptiles because of a misdiagnosis of heartwater could harm a significant source of income for developing exporting countries like Ghana. Consequently, this may also have an impact on conservation efforts in such countries as Ghana, where sustainable natural resources that benefit the populace economically are the principal motivation for conservation.[4] Because of this possible consequence, the correct timely diagnosis of exotic and emerging infectious disease and appropriate control of these diseases are essential. The question of whether snakes are a reservoir for heartwater or not, or even carriers of the potential tick vector, remains unanswered.[1] However, this is becoming more doubtful as viper plague is further characterized, and based on the fact that viper plague is associated with *A. latum* rather than an *Amblyomma* species.[1] The tropical Bont tick (*A. variegatum*), the established vector of heartwater, has been reported on only one imported reptile, a single savanna monitor in Florida.[5] However, reptile tick-origin rickettsias are becoming important causes of emerging diseases and can infect humans. An example of such a rickettsia is *Rickettsia honei,* the causative agent of Flinders Island spotted fever, transmitted by the reptile tick *A. hydrosauri* and now putatively found on three continents—Australia, Asia, and North America.[6–8] The evidence presented here supports the unclassified viper plague rickettsia's being distinct from *E. ruminantium*.[1]

The type-D retrovirus associated with the viper plague rickettsia appears to have a broad host range, which would suggest that this virus

is not a VSW endogenous retrovirus.[9] Detection of retrovirus, other than endogenous retrovirus sequences, in bovine cells by PCR and in VH2 cells supported this being a new virus. The virus causes fusion plaques and apoptosis of cells, making propagation in living cells and separation from cellular membrane components of an intracellular obligate parasite like rickettsia difficult at best. The viper plague agent, and the associated retrovirus, as well as the clinical implications of these infections, merit further investigation.

Acknowledgments

We thank and acknowledge the Centre International de Recherche en Agriculture pour le Développement (CIRAD) for supporting the work performed in Guadeloupe, French West Indies. We are also grateful to Daniel Gonzalez and Ishmael Rosas for providing valuable suggestions, and to the Defence Threat Reduction Agency for supporting the work in part.

References

1. Kiel, J.L., R.M. Alarcon, J.E. Parker, *et al*. 2006. Emerging tick-borne disease in African vipers caused by a *Cowdria*-like organism. *Ann. N. Y. Acad. Sci.* **1081:** 434–442.
2. Martinez, D., N. Vachiéry, F. Stachurski, *et al*. 2004. Nested PCR for detection and genotyping of *Ehrlichia ruminantium*. *Ann. N. Y. Acad. Sci.* **1026:** 1–8.
3. Burmeister, T., S. Schwartz & E. Thiel. 2001. A PCR primer system for detecting oncoretroviruses based on conserved DNA sequence motifs of animal retroviruses and its application to human leukaemias and lymphomas. *J. Gen. Virol.* **82:** 2205–2213.
4. Gorzula, S., W. O. Nsiah & W. Oduro. May 1997. *Survey of the Status and Management of the Royal Python (*Python regius*) in Ghana*. Wildlife Department, Accra & Institute of Renewable Natural Resources. Kumasi.
5. Burridge, M.J. 2001. Ticks (Acari: Ixodidae) spread by the international trade in reptiles and their potential roles in dissemination of diseases. *Bull. Entomol. Res.* **91:** 3–23.
6. Stenos, J., S. Graves, V.L. Popov, *et al*. 2003. *Aponomma hydrosauri*, the reptile-associated tick reservoir of *Rickettsia honei* on Flinders Island, Australia. *Am. J. Trop. Med. Hyg.* **69:** 314–317.
7. Billings, A.N., X.-J. Yu, P.D. Teel, *et al*. 1998. Detection of a spotted fever group rickettsia in *Amblyomma cajennense* (Acari: Ixodidae) in south Texas. *J. Med. Entomol.* **35:** 474–478.
8. Jiang, J., V. Sangkasuwan, K. Lderdthusnee, *et al*. 2005. Human infection with *Rickettsia honei*, Thailand. *Emerg. Infect. Dis.* **11:** 1473–1475.
9. Clark, H.F., M.M. Cohen & P.D. Lunger. 1973. Comparative characteristics of a C-type virus-producing cell line (VSW) and a virus-free cell line (VH2) from *Vipera russelli*. *J. Natl. Cancer Inst.* **51:** 645–657.